MUTATIONS TO SULFONAMIDE RESISTANCE IN *STAPHYLOCOCCUS AUREUS*[1]

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TWO general hypotheses have been postulated to explain the origin of drug-resistant strains of bacteria. These hypotheses are: (1) Resistant cells arise by mutation and are selected when grown in the presence of drugs. (2) Resistance is the result of a specific adaptation of bacteria in response to inhibiting agents.

Resistance of *Escherichia coli* to bacteriophage (LURIA and DELBRÜCK, 1943) resistance of *Staphylococcus aureus* to penicillin (DEMEREC 1945), and resistance of *Escherichia coli* to ultraviolet radiation (WITKIN 1946) have been shown to be the result of mutations in individual cells. These mutant cells and clones formed from them possessed selective advantages in the presence of the destructive agent concerned. It is generally accepted that naturally occurring strains of sulfonamide-sensitive species of bacteria exhibit low tolerance for these drugs (SCHMIDT *et al.*, 1940; FRISCH 1942; SCHMIDT *et al.* 1943; LANDY and GERSTUNG 1945; BERNHEIMER 1946). Resistance to sulfonamides is characteristic of a large number of bacterial cultures isolated from patients who have undergone sulfonamide therapy (FRISCH 1942; SCHMIDT *et al.* 1942; EPIDEMIOLOGY UNIT NO. 22, 1945; LANDY and GERSTUNG 1945; BERNHEIMER, 1946). However, the mechanism by which sulfonamide fastness is acquired is not clearly understood. STRAUSS *et al.* (1941a and b), SESLER and SCHMIDT (1942), KIRBY and RANTZ (1943), and SCHMIDT and SESLER (1943) reported that sulfonamide tolerance is the result of physiological adaptation of bacteria, but their experiments do not give conclusive evidence for this mechanism. LANKFORD *et al.* (1943) and SPICER (1945) concluded that naturally occurring bacterial populations are a mixture of cell types; sulfa drugs act merely as selective agents in the development of resistant strains. No data were given on the origin of these few resistant cells or on their rate of occurrence.

As the mode of origin of sulfonamide-resistant strains of bacteria is subject to debate, it was felt that this phenomenon needed additional clarification. Such information would be valuable in therapy, for if mutation and selection would prove to be the mechanisms involved, doses of sulfonamides should be high enough to inhibit either the few resistant cells that may be present in a sensitive bacterial strain or resistant cells that may arise by mutation. Also,

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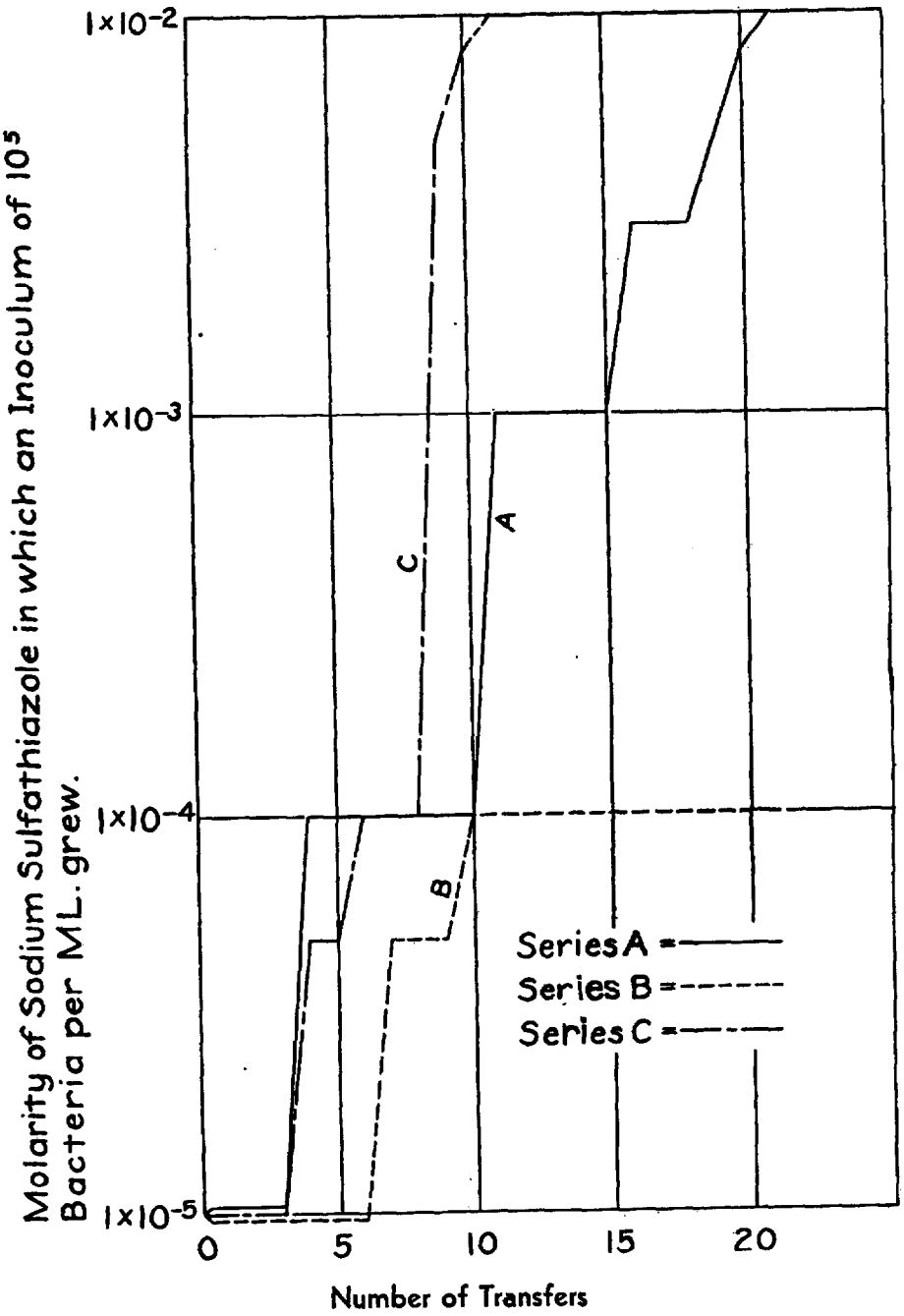

FIGURE 1.—Resistance of *S. aureus* during serial transfer in Sodium Sulfathiazole.

as the action of sulfonamides has been associated with specific cell processes—especially *p*-aminobenzoic acid metabolism—it was hoped that changes in physiological characteristics could be correlated with the change from sensitivity to resistance.

MATERIALS

The strain of *Staphylococcus aureus* used was obtained originally from the NORTHERN REGIONAL RESEARCH LABORATORY, Peoria, Illinois. This same strain, designated as NRRL-313 was used by DEMEREC (1945) in a study of mutations to penicillin resistance.

Sodium sulfathiazole (NaST) was chosen because of its high antibacterial activity and because of its high solubility. As strains resistant to one sulfonamide usually show cross-resistance with most sulfa derivatives, with the exception of sulfamylon (SCHMITH 1943), the experiments were confined to one drug. NaST was prepared in 1×10^{-1} M solution in distilled water and diluted to the desired concentrations.

A semisynthetic casein hydrolysate medium described by STRAUSS *et al.* (1941b) was used in order to avoid sulfonamide antagonists present in ordinary bacteriological media. Twenty-five gms of powdered agar were added per 1900 ml of medium for pouring plates.

DEVELOPMENT OF RESISTANT STRAINS BY SERIAL TRANSFER

Initial attempts to establish resistant strains by isolation of the colonies that developed when a sensitive culture was plated in sulfonamide-agar were precluded by the effects of inoculum size. Large inocula gave complete growth even in agar saturated with NaST. No colonies developed when the number of organisms plated was small enough for inhibition of bacterial growth. Therefore, preliminary experiments were based upon observation of the magnitude and distribution of increases in resistance that occurred during serial transfer in liquid medium containing NaST.

1×10^{-5} M was the maximum concentration of NaST in which an inoculum of 10^2 or 10^3 cells per ml of strain NRRL-313 grew. At each transfer inocula of 10^6, 10^5, 10^4, 10^3, and 10^2 bacteria per ml of culture were tested with a range of 1×10^{-2} to 1×10^{-5} M NaST. Growth was estimated from turbidity after 48 and 72 hours incubation at 37°C. Bacteria for the next transfer were taken from the highest concentration of NaST in which an inoculum of 10^5 cells per ml had grown to almost full turbidity in 72 hours. This inoculum was selected because it was inhibited by the same NaST molarities as inocula of 10^3 and 10^2 cells per ml, yet it was large enough to allow detection of resistant cells. As resistance of the strains increased the higher concentrations of NaST were spaced at closer intervals, and some of the lower concentrations were omitted.

Results of three transfer series, A, B, and C are given in figure 1. A total increase in NaST tolerance of 1,000 times occurred in series A and C, and an increase of ten times occurred in series B. The characteristic resistance of each

resultant strain was maintained through 15 transfers in the absence of NaST, and therefore appeared to be a stable, heritable property.

Increases in resistance appeared as discrete steps that were of different magnitude and were separated by flat plateaus. The number of transfers separating the steps differed both between different series and between successive increases within the same series. It is possible that the steps observed were somewhat forced by the NaST concentrations used. The data do not support this hypothesis, for in nine instances intermediate NaST concentrations were bypassed at a single transfer.

Stepwise increases in resistance occurring at random time during the course of serial transfers, and stability of resistance in the absence of sulfonamides, are better explained by mutation and selection than by physiological adaptation. The resistant mutants that arise in a sensitive strain possess selective advantages when grown in the presence of sulfonamides, and should constitute a large proportion of the inoculum used for the next transfer. Consequently, serial transfer in liquid medium is unsuitable either for estimation of mutation rates or for isolation and study of individual mutants.

DERIVATION OF RESISTANT STRAINS BY COLONY ISOLATIONS

When Seitz filtrates from cultures in NaST were tested, it was observed that a reduction of sulfonamide activity had occurred even when visible growth was not reached. No loss of NaST could be demonstrated by colorimetric titration, but this method may not have given a selective differentiation between biologically active and biologically inactive sulfonamide molecules. Another hypothesis is suggested by the observation of ROSE and FOX (1942) that bacteria exposed to sulfonamides undergo a certain number of divisions before growth is stopped. Production of antagonists during this noninhibited phase of bacterial growth would result in reduction of effective sulfonamide concentrations. The relation of antagonist production to growth also was indicated by the absence of inoculum-size effects when the bacteria tested previously had been exposed to inhibitory concentrations of NaST. CHANDLER and JANEWAY (1939) and LOWELL *et al.* (1941) made similar observations. Utilization of inhibited bacteria therefore should allow the plating of inocula large enough for the detection of rare resistant cells.

The following technique was used for the isolation of resistant bacteria from strain NRRL-313. Each of ten tubes containing ten ml of casein hydrolysate broth was inoculated with approximately 10^2 sensitive bacteria from a 24-hour culture. These tubes were incubated for 48 hours at 37°C. From each of these ten cultures grown from small inocula a 3.5-ml culture containing 1×10^{-4} M NaST was inoculated with 10^6 cells per ml. These NaST cultures had titers between 1 and 2×10^8 cells per ml after 18 hours of incubation at 37°C instead of the 8 to 10×10^8 cells per ml observed in control cultures. These inhibited bacteria were tested in plates containing a final concentration of 3×10^{-5} M NaST. One ml of each undiluted culture was poured into each of three test plates. Three 1-ml portions of a 10^{-6} dilution were plated in agar without NaST for assay of the bacterial titer.

All colonies that developed in NaST agar were counted, and samples from each culture were transferred to 5-ml tubes containing 1×10^{-5} M NaST. Bacteria from strain NRRL-313 grew slowly in this concentration of NaST, while resistant mutants were not inhibited. Thus overgrowth of the resistant cells by more sensitive, faster-growing organisms was prevented. As 1×10^{-5} M represented the minimum inhibitory concentrations of NaST, selection pressure in favor of secondary mutants with still higher resistance should have

TABLE 1

Resistance of mutants from different strains of S. aureus.

MAXIMUM MOLAR CONCENTRATION OF NaST IN WHICH MUTANT GREW*	PARENT STRAIN	SUBSTRAINS FROM NRRL-313		SUBSTRAIN FROM STRAIN 38
	NRRL-313, RESISTANT TO 1×10^{-5} M NaST	STRAIN 33, RESISTANT TO 5×10^{-5} M NaST	STRAIN 38, RESISTANT TO 1×10^{-4} M NaST	STRAIN 67, RESISTANT TO 1×10^{-3} M NaST
$5\text{–}6^{**}\times10^{-5}$	1			
$1\text{–}2\times10^{-4}$	15	19		
$3\text{–}4\times10^{-4}$	1	0	12	
$5\text{–}6\times10^{-4}$		4	4	
$9\text{–}10\times10^{-4}$		7	18	
2×10^{-3}				6
3×10^{-3}				6
4×10^{-3}				5
Total number of independent mutants isolated and tested	17	30	34	17

* Test inoculum 10^3 cells per ml of final culture.

** Variation between different experiments necessitates expression of NaST tolerance as a range for most strains.

been minimized. The resulting cultures were tested by adding approximately 10^3 bacteria per ml to 2-ml broth tubes containing varied NaST concentrations. Resistant strains were established on nutrient agar slants from tubes where complete growth had occurred in concentrations of NaST higher than 1×10^{-5} M.

Similar techniques were used for isolation of highly resistant mutants from strains obtained from NRRL-313. NaST concentrations were increased five times for strain 33, ten times for strain 38, and 100 times for strain 67.

NaST resistance of the mutant strains

Resistance of the substrains isolated is given in table 1. Variation of individual cultures in different tests made it necessary to evaluate resistance in terms of a range of NaST concentrations; the values given represent maximum

tolerance as measured by at least three different tests. Strain NRRL-313, resistant to 1×10^{-5} M NaST, gave mutants resistant to $5\text{–}6\times10^{-5}$, $1\text{–}2\times10^{-4}$, and $3\text{–}4\times10^{-4}$ M NaST. Strain 33, a substrain from NRRL-313 that was resistant to 5×10^{-5} M NaST, gave mutants resistant to $1\text{–}2\times10^{-4}$, $5\text{–}6\times10^{-4}$, and $9\text{–}10\times10^{-4}$ M NaST. Strain 38, a substrain from NRRL-313 that was resistant to 1×10^{-4} M NaST, gave mutants resistant to $3\text{–}4\times10^{-4}$, $5\text{–}6\times10^{-4}$, and $9\text{–}10\times10^{-4}$ M NaST. Strain 67, a substrain from strain 38 that was resistant to 1×10^{-3} M NaST, gave mutants resistant to 2×10^{-3}, 3×10^{-3}, and 4×10^{-3} M NaST. Increases from strain 67 seem small, but these bacteria already had undergone two mutations, each of which had resulted in an increase of ten times in NaST tolerance. Also, the actual amount of sulfonamide

TABLE 2

Test for sulfonamide-resistant colonies in ten "inhibited" cultures of NRRL-313.

CULTURE	TITER OF CULTURES* ($\times10^8$)	COLONIES IN TEST PLATES			TOTAL COLONIES
		PLATE 1	PLATE 2	PLATE 3	
A	1.6	0	0	0	0
B	1.1	13	11	11	35
C	1.5	32	40	39	111
D	1.5	0	0	0	0
E	0.9	0	0	0	0
F	2.3	0	0	0	0
G	2.4	0	0	0	0
H	1.5	2	8	10	20
I	2.8	6	4	3	13
J	2.1	2	2	1	5

* Mean of three assay plates.

involved in changing from 1×10^{-3} to 4×10^{-3} M concentrations is much greater than for any previous step. These results indicate that the few colonies that grow when a large inoculum is plated in NaST agar have increased resistance to NaST. Variants with even higher resistance may be obtained from the strains first isolated.

Origin of resistant bacteria

Whether the resistant variants arise by mutation or by physiological adaptation may be tested by the method proposed by LURIA and DELBRÜCK (1943). If cultures are started from an inoculum small enough to make the inclusion of resistant cells highly improbable, random variation in the occurrence of rare mutations should result in statistically significant fluctuations in the number of resistant cells in different cultures. No significant differences between cultures would be expected if the process involved is one of adaptation.

Data for a typical experiment are given in table 2. The fact that some

cultures contained no resistant cells while other cultures contained many is conclusive evidence that resistance was not the result of physiological adaptation.

The postulate that mutations arise at random time during the growth of cultures was tested by variance analysis of those cultures that gave colonies in each of three test plates (table 3). The omission of cultures with no colonies

TABLE 3

*Variance analysis of the number of colonies per culture.**

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE
Total	14	
Between cultures	4	614.05**
Between plates within cultures	10	8.07

* Data from Table 2, limited to cultures that showed colonies.
** Significant at one percent level.

TABLE 4

Significance of variation in number of resistant cells in different cultures.

CULTURES USED AS PARENT STRAINS	NUMBER OF EXPERIMENTS AVAILABLE FOR STATISTICAL TEST*	SIGNIFICANCE OF BETWEEN-CULTURE MEAN SQUARES		
		NO SIGNIFICANT DIFFERENCES BETWEEN CULTURES	DIFFERENCES BETWEEN CULTURES SIGNIFICANT AT 5% LEVEL	DIFFERENCES BETWEEN CULTURES SIGNIFICANT AT 1% LEVEL
NRRL-313	2	0	0	2
Strain 33	4	0	0	4
Strain 38	2	0	1	1
Strain 67	1	0	0	1
Totals	9	0	1	8

* Analysis limited to experiments with three or more cultures that gave resistant colonies on each of three plates per culture.

minimizes differences between cultures. Nevertheless, variation in the number of resistant cells per culture was highly significant. The results for nine independent experiments are given in table 4. Variation between cultures was significant at the one percent level in eight experiments and significant at the five percent level in one experiment. This highly significant fluctuation in the number of resistant cells in different cultures started from a common source is strong evidence that resistant bacteria arise by mutation.

Mutation rates

The rate of mutation from sensitivity to resistance was estimated from the percentage of cultures not giving resistant colonies (LURIA and DELBRÜCK 1943). Data for strain NRRL-313 and three substrains from NRRL-313 are given in table 5. Different classes of resistant mutants were pooled to form the total for each individual strain. The four strains tested all gave similar mutation rates, with a range of 2×10^{-9} to 4×10^{-10} per bacterium per generation. This close agreement between strains, even though some had undergone two previous mutations, suggests a large number of different mutations to NaST resistance.

The minimum number of mutations concerned probably is five; the maximum number may be considerably larger. The minimum figure is based on

TABLE 5

Mutation rates of different strains of S. aureus.

PARENT STRAIN	NaST RESISTANCE OF PARENT STRAINS	TOTAL NUMBER OF CULTURES	NUMBER OF CULTURES WITHOUT MUTANTS	FRACTION OF CULTURES WITHOUT MUTANTS	MEAN NUMBER OF BACTERIA TESTED PER CULTURE ($\times10^8$)	MUTATION RATE PER BACTERIUM PER GENERATION
NRRL-313	1×10^{-6}	50	34	0.68	6.9	4×10^{-10}
Strain 33	5×10^{-6}	40	12	0.30	4.3	2×10^{-9}
Strain 38	1×10^{-4}	40	19	0.48	6.4	8×10^{-10}
Strain 67	1×10^{-3}	40	21	0.53	5.0	9×10^{-10}

strain 67, which gave three classes of mutants after having undergone two previous mutations to NaST resistance. Except for increased *p*-aminobenzoic acid production, no criterion was found for differentiation of mutants from different strains. Equal magnitude of resistance changes cannot be used, for it is impossible to determine how the same mutation may affect the phenotype when it occurs in cells of different genetic constitution.

PRODUCTION OF *p*-AMINOBENZOIC ACID BY RESISTANT STRAINS

According to the WOODS-FILDES hypothesis (WOODS 1940) that sulfonamides act through competition for an essential metabolite—specifically, *p*-aminobenzoic acid (PAB)—increased PAB synthesis could account for increased bacterial resistance. LANDY, et al. (1943) reported that sufficient PAB was present in the filtrate of a sulfonamide-resistant culture of *Staphylococcus aureus* to explain the level of resistance observed. However, LANDY and GERSTUNG (1945) found that while a high percentage of sulfonamide-fast strains of gonococci showed increased PAB synthesis, some resistant strains produced very little PAB. Conversely, some sensitive strains produced more PAB than others. Therefore, while many resistant strains showed increased production

of an antagonist identified as PAB, other mechanisms of resistance also were involved.

Sulfonamide antagonists in filtrates of full-grown cultures of strains NRRL-313, 33, 38, and 67, of strains A, B, and C from the serial transfers, and of a sample of strains from each class of mutants isolated from plates were tested by the following methods: (1) ability of filtrates to antagonize NaST, thereby allowing growth of sensitive staphylococci; (2) ability of filtrates to replace PAB as a growth factor for *Acetobacter suboxydans* (LANDY and DICKEN, 1942); and (3) ability of filtrates to support growth of a *p*-aminobenzoicless strain of *Neurospora crassa*[4] (THOMPSON *et al.* 1943). Presence of PAB in filtrates of

TABLE 6

p-aminobenzoic acid production by sulfonamide-resistant mutants of Staphylococcus aureus.

MUTANTS FROM STRAINS WITH LOW PAB PRODUCTION				MUTANTS FROM STRAINS WITH INCREASED PAB PRODUCTION			
FROM NRRL-313, RESISTANT TO 1×10^{-5} M NaST		FROM STRAIN 33,** RESISTANT TO 5×10^{-5} M NaST		FROM STRAIN 38,*** RESISTANT TO 1×10^{-4} M NaST		FROM STRAIN 67,**** RESISTANT TO 1×10^{-3} M NaST	
MUTANTS TESTED		MUTANTS TESTED		MUTANTS TESTED		MUTANTS TESTED	
NaST RESISTANCE	INCREASE IN PAB PRODUCTION	NaST RESISTANCE	INCREASE IN PAB PRODUCTION	NaST RESISTANCE	INCREASE IN PAB PRODUCTION	NaST RESISTANCE	INCREASE IN PAB PRODUCTION
$5-6\times10^{-5}$	−*	$1-2\times10^{-4}$	−	$3-4\times10^{-4}$	−	2×10^{-3}	−
$1-2\times10^{-4}$	+	$5-6\times10^{-4}$	+	$5-6\times10^{-4}$	−	3×10^{-3}	−
$3-4\times10^{-4}$	+	$9-10\times10^{-4}$	+	$9-10\times10^{-4}$	−	4×10^{-3}	−

* −Indicates no increase over parent strain; +indicates increase over parent strain. Increased PAB production was by the same amount whenever it occurred.

** Colony isolation from NRRL-313 with same PAB production as strain NRRL-313.

*** Colony isolation from NRRL-313 with increased PAB production.

**** Colony isolation from strain 38 with same PAB production as strain 38.

S. aureus cultures is indicated by the fact that positive results were obtained with all assay procedures used.

A summary of the data on PAB production by different strains is given in table 6. Some resistant strains synthesized more PAB than the sensitive NRRL-313 strain; others did not. Mutants from NRRL-313 that were resistant to $5-6\times10^{-5}$ M NaST showed no increase in antagonist production; those resistant to NaST concentrations of 1×10^{-4} M and higher produced more antagonists than the parent strain. When strain 33 was used as the parent strain, mutants resistant to 1×10^{-4} M NaST showed no increased production of antagonist; mutants resistant to NaST molarities of 5×10^{-4} or higher showed increased antagonist production. Strains 38 and 67 already had undergone one mutation that resulted in formation of higher amounts of

[4] This strain of Neurospora was kindly supplied by DR. E. L. TATUM of YALE UNIVERSITY, New Haven, Connecticut.

PAB. None of the mutants from these strains showed further increases in production of antagonist. Mutants with increased PAB production fall into two classes when classified on the basis of NaST tolerance: those mutants with resistance increased by 10–20 times and those with resistance increased by 30–40 times. Increase in PAB production always was by the same amount. Therefore, while these mutations differed in effect on NaST tolerance, they had a common effect on PAB production.

All increases in production of sulfonamide antagonists were by a factor of ten times as measured by antagonism of NaST for sensitive *Staphhylococcus aureus*, by a factor of twenty times as measured by *Acetobacter suboxydans*, and by a factor of 30 times as measured by *p*-aminobenzoicless *Neurospora crassa*. An increase of 10–30 times in PAB production explained changes in sulfonamide tolerance of similar magnitudes when mutations affected both NaST resistance and PAB synthesis simultaneously. While it was a factor, production of antagonist did not account for the increases in resistance observed in all mutant strains, because mutants with increased NaST resistance and with no increase in PAB production were obtained from strain 38 and strain 67 (table 6). Also, strains with NaST resistance ten times that of NRRL-313 could be obtained by two successive mutations that did not affect PAB production.

Conclusions based on filtrates may be misleading. The use of both filtrates and culture extracts (MACLEOD, 1940) offers another approach, but it is questionable whether or not extracts correctly represent conditions in living cells. Also, it is difficult to determine if measurement of PAB in fully grown cultures gives an accurate estimate of conditions existent during the early stages of growth.

DISCUSSION

Discrete stepwise increases in resistance that occur at random time during serial transfer in NaST, and significant variation in the number of resistant cells in independent cultures, indicate mutation and selection as the mechanisms by which sulfonamide-resistant strains of *Staphylococcus aureus* originate. The same conclusion has been reached concerning resistance of *Escherichia coli* to bacteriophage (LURIA and DELBRÜCK (1943), resistance of *Staphylococcus aureus* to penicillin (DEMEREC 1945), and resistance of *Escherichia coli* to ultraviolet radiation (WITKIN 1946). Mutation and selection therefore appear to be the mechanisms by which resistance to antibacterial agents originates in many species of bacteria. In like manner, development of sulfonamide resistance in most species of microorganisms probably occurs by the same mechanisms as in *Staphylococcus aureus*.

SESLER and SCHMIDT (1942) reported experiments on serial transfer of pneumococci in sulfonamides. The data published showed stepwise increases in resistance that occurred at random time during transfer, but they concluded that the mechanism involved was one of adaptation, and that the steps were forced by the concentrations of sulfonamides used. Nevertheless, in the nine transfer series SESLER and SCHMIDT reported, there were seven instances where increases in resistance bypassed one and often two intervening concentrations of sulfonamide at one transfer. Also, adaptation does not explain the random-

ness in time of occurrence of the rises. Identical results obtained in the transfer experiments reported here were interpreted as constituting evidence for origin of resistance through mutation and selection.

Tests of the hypothesis that resistant bacteria arise by mutation should be made with organisms that have been grown in absence of inhibiting agents. This was impossible with sulfonamides because of inoculum-size effects. Nevertheless, validity of the data for determining the importance of mutations was not affected by the techniques used, for many cultures contained no resistant cells. This is conclusive evidence that physiological adaptation was not the mechanism by which resistant bacteria were developed. Preliminary exposure to sulfonamides could result in a serious error because of differential selection if several discrete mutations were involved at each step, for statistical analyses of the number of resistant cells in different cultures would be biased if the more resistant mutants had higher selective values. Clone size would reflect different resistance levels rather than random occurrence of mutations. The data gave no indication that such a condition existed, for highly significant variation between cultures was found in experiments where all mutants isolated showed identical resistance. Also, in some cases the highest number of colonies per culture was associated with low resistance.

Mutation rates were calculated from the percentage of cultures containing no resistant cells. Computation of mutation rates from the actual number of mutants present in the cultures could not be made from these data because of selection pressures existent during preliminary exposure to NaST.

The majority of mutants from NRRL-313 had NaST tolerance increased by about ten times, a few had NaST resistance increased by 30–40 times, and a few had NaST resistance increased by five times (table 1). All mutants with NaST tolerance increased by ten or by 30–40 times showed increased production of a sulfonamide antagonist capable of replacing *p*-aminobenzoic acid as a growth factor for *Acetobacter suboxydans* and for a *p*-aminobenzoicless strain of *Neurospora crassa*. Strains already resistant to ten times as much NaST as the parent strain NRRL-313 gave mutants with resistance increased by a second factor of ten times. Antagonist production was not altered by the second mutation. Therefore, some colony isolations from NRRL-313 might be expected to show an increase in resistance of ten times and no increased antagonist production. Such strains were not found. Either an insufficient number of colonies was tested, or the action of the genes concerned is conditioned by the genotype in which they occur. For example, mutations that increased sulfonamide tolerance by five times when they occurred in NRRL-313 may have given increases of ten times in strain 38. Another possibility is that mutations in strain 38 affect metabolic systems that are of importance in sulfonamide resistance only when PAB metabolism has been altered.

Several mechanisms of sulfonamide action also are suggested by the fact that the antagonist titer of filtrates depends on the method of assay used. Tests with *Staphylococcus aureus*, *Acetobacter suboxydans*, and *Neurospora crassa* each gave different results. NaST resistance of the parent strain NRRL-313 probably is the summation of several effects. Therefore, variation in PAB pro-

duction by a factor of 30 times as measured by Neurospora could show an increase of only ten times as measured by Staphylococci because some antagonists in the filtrate of NRRL-313 are effective in counteracting NaST but do not replace PAB as a growth factor for *p*-aminobenzoicless *N. crassa*. Antagonists other than PAB that may be present in the filtrate of NRRL-313 were not increased during the change from sensitivity to resistance, because tests with *S. aureus* gave the same anti-sulfonamide value for filtrates from all cultures with increased antagonist production.

Selection of resistant mutants probably is the primary factor responsible for development of drug-resistant strains that frequently occurs when a low dosage of sulfonamide is used either for prophylaxis or for long courses of treatment (EPIDEMIOLOGY UNIT No. 22, 1945; BERNHEIMER 1946). Single-step mutants with high resistance occur at a low rate; for none were isolated either by DEMEREC (1945) or during this investigation. Bacteria with low drug tolerance were found to be relatively common, and if established as a strain, subsequently gave mutants with higher resistance. Therefore, if therapeutic doses are large enough to inhibit the primary mutants that may occur, development of highly resistant bacterial strains through selection of secondary mutants will be prevented. Treatments should be as intensive as possible in order to preclude development of resistant strains of bacteria.

SUMMARY

The mode of origin of sulfonamide-resistant strains of *Staphylococcus aureus* was studied by means of serial transfer in the presence of sodium sulfathiazole and by isolation of colonies from plates containing sodium sulfathiazole.

Stepwise rises in resistance that occurred at random time during serial transfer, and significant variation between similar cultures in the number of resistant cells they contained, indicated mutation and selection as the mechanisms by which resistant strains of bacteria were developed.

Mutant strains with moderate resistance gave rise to variants with still higher sulfonamide tolerance. Bacteria capable of growing in saturated solutions of sodium sulfathiazole were obtained by successive mutations to resistance.

Calculations based on the proportion of cultures that failed to show resistant cells gave rates of mutation from sensitivity to resistance that ranged from 2 times 10^{-9} to 4 times 10^{-10} per bacterium per generation.

At least five different mutations affect the sulfonamide resistance of *S. aureus*. Only one or possibly two mutations are associated with increased production of *p*-aminobenzoic acid. Therefore, it appears as if several cell mechanisms are sensitive to sulfonamides and are altered by mutations.

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